CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231, on December 4, 1984.

By Gilbert G. Kovelman, Reg. No. 19,552

December 4, 1984
Date of Signature

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
RICHARD H. TULLIS	Group Art Unit: 127
Serial No. 314,124	Examiner: Martinell
Filed: October 23, 1981	
For: OLIGONUCLEOTIDE THERAPEUTIC AGENT AND METHODS OF MAKING SAME	

Los Angeles, CA 90010
December 4, 1984

AMENDMENT

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

This amendment is in response to the Office Action mailed June 4, 1984, regarding the above-identified application. The shortened statutory period for response expired on September 4, 1984, and a three-month extension of time under 37 C.F.R. 1.136(a) is requested, the requisite fee of $175 being enclosed. In response to the aforementioned Office Action, please amend the above referenced application as follows:

IN THE CLAIMS:

Please amend Claim 25 as follows:

C1

25. (Twice Amended) The method of Claim 24 wherein the oligonucleotide base sequence comprises:

GTGTAGCAGTAR$_1$CCR$_2$GCGCACCA,

and wherein R_1 is G or T and R_2 is G or T.

REMARKS

This application has been carefully reviewed in light of the Office Action dated June 4, 1984, and in view of a telephone interview with Examiner Martinell that took place on November 15, 1984. The Examiner's careful attention in reviewing the application and his time and helpful suggestions during the telephone interview are greatly appreciated.

Regarding the previous restriction requirement, Applicant notes that Claims 31-39 have been rejoined and are to be examined on the merits, leaving only Claims 40-52 withdrawn from consideration. The rejoining of Claims 31-39 is much appreciated. As to Claims 40-52, Applicant is cognizant of his right to petition the Commissioner after final action on or allowance of claims to the invention elected, but prior to appeal, pursuant to 37 C.F.R. 1.144.

The Examiner has objected to the specification under 35 U.S.C. 112, as failing to provide an enabling disclosure, and has rejected Claims 1-4, 6-15, 18-25, 27-37, 39 and 53 for the same reason. The essence of these rejections is that these claims and the specification include RNA-RNA hybridization, which the Examiner asserts cannot occur in vivo

because an oligoribonucleotide probe capable of hybridization would not be taken into the cytoplasm of a cell, where it must appear in order to hybridize as claimed.

Two references are cited by the Examiner as supporting this rejection, namely Summerton (especially pages 84-85) and Befort et al. (page 184). As Applicant explained during the telephone interview, however, these two references in no way suggest that RNA is not taken up by a cell into the cytoplasm. In fact, both of the cited references indicate that RNA is actually well taken up by cells.

Page 84 of the Summerton reference succinctly confirms Applicant's position: "RNA is taken up in substantial quantity by animal cells." The Examiner's concerns, as expressed during the telephone interview, seem to be that RNA would not be taken up because it is single-stranded. But again, Summerton teaches the opposite. In discussing the uptake of single-stranded DNA's, Summerton states on page 184:

> . . . until recently (1971) single-stranded DNA was also widely reported to transform bacteria very poorly and what transformation did occur was often attributed to reannealed duplex material contaminating the single-stranded preparations. However, since 1971 bacterial transformation by single-stranded donor has been thoroughly substantiated and shown to occur with reasonably high efficiency. (Emphasis added).

Summerton then goes on to discuss a study by Herrera et al., which illustrates the uptake of t-RNA by animal cells. Although the Examiner has attempted to disqualify t-RNA as a useful example because of its high degree of secondary structure, Summerton still states that single-stranded nucleotides, whether RNA or DNA derived, are taken up substantially by cells and can actually cause significant gene transformation.

Similarly, Befort et al. supports Applicant's position, as shown by the statement on page 184: "However, our results show that all RNA's, modified or not, enter the cells and are preferentially located at the microsomal level." This portion of the article discusses the significantly improved uptake of modified RNA's, possibly because they are resistant to endogenous nucleases. Again, this strongly supports enablement of Applicant's disclosure, since Applicant's oligonucleotides are first protected (modified), preferentially by converting them into a phosphotriester form. In fact, in Applicant's last response, the claims of the present invention were amended so that all claims include converting the oligonucleotide be used as the probe to a more stable form.

In addition to the aforedescribed references cited by the Examiner, the two Stebbing references cited by Applicants in their disclosure statement also show clearly that oligonucleotides are significantly taken up by cells. For example, a discussion of the in vivo antiviral effect of single-stranded RNA's can be found on page 302 of the Stebbing article entitled "The Design of Antiviral Agents Based On Strategic Sequences in Viral RNA and Antiviral Effects of Single-Stranded Polynucleotides," where it is stated that ". . . Protection has been observed with these single-stranded polynucleotides against infection of mice by SFV. . ." On page 303 of the same article it is stated that intraperitoneal injection of polynucleotides (single stranded RNA's) protected up to half of the mice injected with MM virus.

The second Stebbing reference, entitled "Cellular Uptake And In Vivo Fate Of Polynucleotides" specifically

addresses the issue at hand. On page 480, the article states that:

> [d]irect evidence that polynucleotides can enter cells comes from the repeated observation of the infectivity of purified viral RNA's (Koch and Bishop, 1968; Pagano, 1970). . . . Another interesting demonstration of uptake of a functional RNA concerns the ability of RNA extracted from human fibroblasts treated with Poly (I), Poly (C) to cause production of human interferon in chick fibroblast cells (Reynolds & Pitha, 1974). . . . These results (Reynolds and Pitha, 1974) indicate that the material causing production of human interferon in chick cells was messenger RNA for interferon from the human fibroblasts.

The Examiner has stated that t-RNA, because of its high degree of secondary structure, is not helpful in showing uptake of single-stranded RNA's. This discussion, however, deals with uptake of m-RNA, which has less secondary structure than t-RNA.

A discussion on page 490 of the same Stebbing article also shows in vivo uptake: "Several studies indicate that the relative effect of polynucleotides in vitro against reverse transcriptases is similar to their effects on virus infection in vivo, implying a direct effect on the enzyme in infected cells (Arya et al., 1975, 1976b; Stebbing, 1979).

Finally, the Miller et al. reference entitled, "Effects of a Trinucleotide Phosphotriester, $G^{m}p(Et)G^{m}p(Et)U$, on Mammalian Cells in Culture," cited in Applicant's Disclosure Statement, provides firm proof that an oligonucleotide, in this case a phosphotriester protected trimer, is taken up by cells in vivo. In their study, Miller et al. achieved significant disruption of protein synthesis in hamster fibroblast cells, thereby showing uptake of the oligonucleotide by the cells. The study stated on page 1995: "Since the calculated triester concentration within the cell far exceeds the extracellular concentration, the

uptake data suggested the triester is metabolized by the cells."

Applicant submits that the above references show that an oligonucleotide used in accord with his invention will appear in the cytoplasm of a cell, and will therefore be capable of hybridizing to the cell's m-RNA as specified. In Applicant's response dated February 1, 1984, to the previous Office Action, dated August 8, 1983, and the accompanying affidavit of Robert N. Bryan, Applicant demonstrated the utility of the invention in vitro, and cited authority stating that an invention need not be shown to work in vivo in order to be patentable. With the above cited references suggesting that Applicant's invention will work in vivo if it works as claimed in vitro, which it has been shown to do, Applicant requests that the non-enablement rejection of the specification and claims under 35 U.S.C. 112 be withdrawn.

It has been quite properly suggested by the Examiner that Claim 25 should be again amended, due to an inadvertent failure in the last response to underline the added matter. In making the suggested amendment, Applicant assumes that although the matter was not added due to lack of underlining, the matter in the claim as originally filed was removed, as it was properly enclosed in brackets. Therefore, Applicant's present amendment is confined to merely adding the matter which was not underlined in the last amendment.

On page 6 of the Office Action, the Examiner has rejected all claims remaining in the application for examination under 35 U.S.C. 103 as being unpatentable over Itakura et al. in view of either one of Paterson et al. or Hastie et al. On page 7, all remaining claims were also rejected in view of the above references, and further in view

of Anderson et al.

The Examiner's rejections are essentially repeated for reasons given in the last office action, which can be summarized as follows. Itakura et al. teaches the synthesis of oligonucleotides with essentially all of the structural features of those of the claims. Paterson et al. and Hastie et al. teach hybrid-arested translation. It is therefore obvious, the Examiner asserts, to use oligonucleotides similar to those in Itakura et al. to accomplish hybrid-arrested translation as taught by Paterson et al. and Hastie et al. Anderson et al. is added to show introduction of oligonucleotides into cells for hybridization.

While the references cited by the Examiner generally show the synthesis of oligonucleotides and hybrid-arrested translation, they are fundamentally different than the oligonucleotides used in Applicant's invention, as will be explained below.

The most fundamental distinction between the references cited by the Examiner to show hybrid-arrested translation (Paterson et al. and Hastie et al.) and Applicant's invention is the size of the nucleic acid segments being used to accomplish translation arrest. Paterson et al. and Hastie et al. use polynucleotides, somewhere between 500 and 1000 bases in length, to arrest translation. Applicant uses oligonucleotides, on the order of 20 bases long. This distinction is extremely important for two reasons. First, long polynucleotides can not hybridize spontaneously at body temperature, which is necessary if a therapeutic agent is to be used in vivo in a living animal. Second, long polynucleotides can not be taken up by cells without treating the cells and thereby causing damage, and even when cells are so

treated, the uptake of polynucleotides is extremely low.

Regarding the inability of large polynucleotides to hybridize spontaneously at body temperature, Applicant addressed this issue in his last amendment on page 16. It was explained that large polynucleotides have too high a melting point to hybridize spontaneously at body temperature. In the present Office Action, the Examiner states that Applicant's prior discussion of melting point actually supports the rejection because a higher melting temperature for a heteroduplex means a more stable hybrid, and thus one that would be even more effective in blocking translation than a heteroduplex with a lower melting temperature and hence a lower stability.

While the Examiner's statement is true in the abstract, it can not be applied to a probe that must work in vivo. Generally, a polynucleotide having a higher melting point will form a more stable hybrid. The problem is that a polynucleotide having a very high melting point will never be able to hybridize at all at body temperature, 37°C. A polynucleotide or oligonucleotide will most effectively hybridize at approximately 25°C below its melting point. Thus, if a polynucleotide has a melting temperature of 100°C, it will only hybridize specifically at approximately 75°C, which of course is much higher than body temperature, 37°C. Once such a polynucleotide forms a heteroduplex, that heteroduplex would indeed be more stable than one formed using a nucleotide having a lower melting temperature. But such a heteroduplex could never be formed spontaneously at body temperature using a polynucleotide such as the ones used in Paterson et al. and Hastie et al. because its melting temperature and, therefore, the temperature at which it will

specifically hybridize, is too high. Thus, the fact that Applicant's invention uses small oligonucleotides that will hybridize spontaneously and specifically at body temperature is crucial when making a comparison to the experiments conducted in Paterson et al. and Hastie et al.

Regarding the inability of large polynucleotides to be taken up by cells without causing damage to the cells, Applicant does not purport to state that the introduction of large polynucleotides into cells, in and of itself, causes cell damage. The Examiner apparently attributes such a statement to Applicant based on a statement made in Applicant's last amendment. Applicant here seeks to explain and clarify his position.

It is not the actual entry of large polynucleotides into cells which causes damage. Rather, it is the treatment that must be applied to the cells to enable them to take up such large strands at all. For example, in Marx, "Gene Transfer Moves Ahead" Science 210, 1334-1336 (1980), it is noted that Graham and van der Eb observed in 1973 that precipitation of DNA with calcium phosphate greatly enhanced gene uptake by cells. A mammalian cell transformation study by Axel, Silverstein and Wigler is also reported, in which the researchers used calcium phosphate to enhance uptake, and still induced transformation in only about 1 in 100,000 or 1,000,000 cells. Calcium phoshate treatment could never be used in a living organism and, even if it could be, the uptake of the nucleotide (as evidenced by the extremely low transformation rate) is much to low for a therapeutic agent to be effective. A copy of the Marx article is enclosed.

Similarly, the Summerton reference, mentioned by the Examiner in the most recent Office Action, reports a study by

Fox et al. (1969) in which an extremely low genetic transformation rate of 1 in 10,000 is observed. The Examiner cites Summerton as showing that large DNA strands do not damage cells when introduced. However, in light of the above discussion, Applicant does not assert the the introduction itself causes cell damage.

To briefly summarize Applicant's position regarding large polynucleotides, the treatment (such as calcium phosphate) that must be used to obtain their uptake into cells causes cell damage and could not be used in a living organism. Even when such treatment is used, cell uptake is extremely low.

On the other hand, small oligonucleotides like those used in Applicant's invention are readily taken up by cells without extraneous treatment. The Miller et al. (1977) reference shows excellent uptake of a trinucleotide ethyl phosphotriester under physiological salt conditions at 37°C, as evidenced by the abstract on page 1988: "The triester is readily taken up by transformed Syrian hamster fibroblasts growing in monolayer." Similarly, the Zamecnik and Stephenson reference, in the abstract on page 280, states that a tridecamer "enter[s] the chick fibroblast cells, hybridize[s] with the terminal reiterated sequences at the 3' and 5' ends of the 35S RNA and interfere[s] with one or more steps involved with viral production and cell transformation."

In his previous response, Applicant stated that it was thought that long polynucleotides would be needed to cause hybrid arrested translation. In the present Office Action, the Examiner questions this statement because Applicant cited no authority. The basis of this statement is

that other than the Miller et al. and Zamecnik and Stephenson references discussed above, Applicant is not aware of any other work prior to his filing date in which short oligonucleotides were successfully employed to cause hybrid-arrested translation. All work on hybrid-arrested translation was performed using long polynucleotides, as in Paterson et al. and Hastie et al. While the Miller et al. and Stephenson and Zamecnik references used short oligonucleotides, there are important differences between these references and Applicant's invention, which will be discussed in detail below.

Regarding the Examiner's citation of Anderson et al. to show introduction of oligonucleotides into cells, Applicant still maintains that this reference is not pertinent to his invention. The Examiner states in the present Office Action that Anderson et al. is not cited to show microinjection of oligonucleotides into cells, but merely as a method of introducing oligonucleotides for hybridization as discussed in view of the primary and secondary references. But it is essential to note that Anderson et al. injected large DNA fragments in the nuclei of cells, a manner in which Applicant's invention can not function if it is to work in vivo. If the DNA fragments in Anderson et al. were introduced into cells in the manner of Applicant's procedure, the Anderson group experiment would have failed becasue such large fragments would not be taken up by the cells, as discussed above. Anderson et al. is therefore not relevant to Applicant's invention as showing a method of introducing oligonucleotides into cells.

On page 8 of the present Office Action, the Examiner has rejected all remaining claims under 35 U.S.C. 103 as being unpatentable over Itakura et al. in view of either one

of Paterson et al. or Hastie et al. as applied to claims 1, 3-28, 30-39 and 53 above, and further in view of Pluskal et al., Pitha (CRC Press), Befort et al., Arya et al. (Molec Pharmacol. or BBRC), Summerton, Tennant et al., Miller et al. (Biochem. 16,1988), Stephenson et al., Zamecnik et al., or Stebbing et al. Each of the tertiary references, the Examiner asserts, discusses the introduction of oligonucleotides into cells. It would therefore be obvious, he concludes, for the ordinary skilled artisan to introduce oligonucleotides of Itakura et al. into cells by the method of any one of the tertiary references to achieve the hybridization of either secondary reference. All of the remaining claims were also rejected under 35 U.S.C. 102 as being clearly anticipated by any one of Stebbing, Zamecnik et al. or Stephenson et al.

Addressing the rejection under 35 U.S.C. 103, it has previously been pointed out that the hybrid-arrested translation disclosed in Paterson et al. and Hastie et al. employed long polynucleotides and, therefore, does not render the present invention obvious. Even though the tertiary references broadly suggest introduction of oligonucleotides into cells, combining these references with the primary and secondary references still does not render Applicant's invention obvious because Applicant uses short oligonucleotides for hybridization. Even more importantly, perhaps, Applicant hybridizes his oligonucleotides to the coding region of an m-RNA in order to inhibit protein synthesis, which was not known in the prior art. In the amendment filed previously by Applicant, the claims were amended so that all claims involve targeting the oligonucleotide against the coding region of an m-RNA.

Regarding the rejection under 35 U.S.C. 102, the fact that Applicant's oligonucleotides hybridize with the coding region of an m-RNA is fundamental in distinguishing Applicant's invention from the work performed in the Zamecnik and Stephenson and Stephenson and Zamecnik references, as well as from the two Stebbing articles. The oligonucleotides in Zamecnik and Stephenson are not specific. Zamecnik and Stephenson used a tridecamer to interfere with the replication of the Rous Sarcoma Virus by preventing circularization. Protein synthesis does not require circularization, and therefore, their method will not function generally as a protein synthesis inhibitor. The circumstance that protein synthesis was inhibited in their experiment was fortuitous.

Further, it is most likely that the Zamecnik and Stephenson method is non-specific because it hybridizes to the 5' non-coding region. Several different m-RNAs might have the same sequence in this region, meaning all of these m-RNAs will be blocked. In addition, the 5' non-coding region may vary within a single type of m-RNA, without a change in gene expression, meaning that the Zamecnik and Stephenson oligonucleotide might be too specific; it would not block an m-RNA containing the desired gene expression, but an alternative 5' non-coding region.

Applicant's oligonucleotide, on the other hand, is designed to be complementary only to the particular section of the M-RNA strand that codes for the particular protein, the sythesis of which is sought to be inhibited. This is not even remotely suggested by either the Zamecnik and Stephenson references or the two Stebbing references.

Clearly it was not obvious at the time of Applicant's invention to design an oligonucleotide to hybridize to

the coding region of an m-RNA. This is because of the fairly high degree of secondary structure of the coding region of m-RNA, as shown by a number of references. Holder and Lingrel, "Determination of Secondary Structure in Rabbit Globin Messenger RNA by Thermal Denaturation", Biochem 14:19, 4209-4215 (1975), discloses that 58-68% of the bases in Globin m-RNA are involved in helical structure. On page 1026 of Agarwal et. al., "A General Method for Detection and Characterization of an mRNA using an Oligonucleotide Probe", J. Bio. Chem. 256:2, 1023-1028 (1981), it is stated that "secondary structure in certain regions of an RNA molecule may interfere with efficient hybridization of oligonucleotides." Van et al., "Comparative Studies on the Secondary Structure of Ovalbumin mRNA and cDNA", Biochem. 16:4090 (1977), concludes that 41% of the bases of ovalbumin m-RNA are involved in secondary structure. Copies of the Holder and Lingrel and Agarwal et al. references are enclosed. A reprint of the Van et al. article was not available to Applicant at the time of filing of the Amendment, but can be furnished at a later date if desired by the Examiner.

The considerable degree of secondary structure of the coding region of m-RNA makes it unobvious to target an oligonucleotide to that region for two primary reasons. First, the oligonucleotide might not hybridize to a sequence in a region of high secondary structure without prior denaturation of the m-RNA. This is a logical assumption because bases involved in secondary structure are by definition already hydrogen-bonded, and would not be available for hybridization to an incoming probe. Under this hypothesis, it would be necessary to first heat the m-RNA (to denature the portions involved in secondary structure) before intro-

duction of the oligonucleotide probe. This would be impossible, of course, in a situation where the probe is to be used in a living organism. Hybridization must occur spontaneously at body temperature.

Second, it would be thought that a short hybridized oligonucleotide, and therefore one with a low binding energy, might be stripped off as the ribosome moves down the m-RNA molecule during protein synthesis. This is also a logical assumption, since the ribosome manages to proceed through the secondary structure of the m-RNA molecule unhindered during normal protein synthesis. A short oligonucleotide of the same number of bases as might be involved in secondary structure at a given point on the m-RNA molecule might well be stripped off during protein synthesis, since a similar hydrogen-bonded section of m-RNA molecule must be opened up for translation to proceed.

Hence it was certainly unobvious at the time of Applicant's invention to target a short oligonucleotide to the coding region of an m-RNA molecule, known to have significant secondary strucure, when the only successful hybrid-arrested translation had occurred using either long polynucleotides or short oligonucleotides targeted to non-specific base sequences in regions of the RNA molecule not involved in secondary structure.

The Examiner has rejected Claims 24 and 25 under 35 U.S.C. 103 as being unpatentable over Itakura et al. in view of either one of Paterson et al. or Hastie et al. and in view of Anderson et al. as applied to claims 1, 3-28, 30-39 and 53 above and, further, in view of Applicant's admitted state of the prior art on page 18 of the instant application. This rejection was repeated for the reasons given in the last

Office Action, which were that since the amino acid sequence for FSH is old, it would be obvious for the skilled artisan to construct an oligonucleotide for use as discussed in the previous rejections, the sequence of which could be deduced from the admittedly known amino acid sequence of FSH by the method taught by Itakura et al. On page 8 of the present Office Action, the Examiner also rejects Claims 22-25, 28 and 30 for essentially the same reasons.

Claims 22-25, 28 and 30 are all dependent, directly or indirectly, on independent Claim 20. As discussed in great detail above, Claim 20 is patentable over the prior art because it was not heretofore known or obvious to target a small oligonucleotide to the coding region of an m-RNA to specifically inhibit protein synthesis. Applicant does not claim as novel the method of determining the particular sequence in an m-RNA molecule that codes for an amino acid in a protein based on knowledge of the amino acid sequence. Claims 22-25, 28 and 30 all incorporate the patentable subject matter of Claim 20, and are therefore patentable for the same reasons expressed in the previous discussion of Claim 20, even though the amino acid sequence for FSH is known in the prior art.

Some additional art has recently been evaluated by Applicant and his attorney and will be submitted shortly to the Examiner by way of a Supplemental Disclosure Statement. It is requested that the further examination of this application on the merits be deferred until the Examiner has received and reviewed this additional art. Applicant believes, however, that the additional art is no more relevant to the invention than the references previously discussed.

In the "List of Prior Art Cited by Applicant" sec-